expenditure by K^+ efflux down the K^+ gradient was insufficient to account for the glycine influx at the glycine-concentration ratio maintained. The other form of the hypothesis is that there is a linked entry of Na^+ and amino acid, with the energy from Na^+ influx down its chemical-activity gradient furnishing the energy for the transport of glycine against its gradient (Riggs *et al.*, 1958). The dependence of glycine entry in Na_o^+ (Kromphardt *et al.*, 1963; Vidaver, 1964) and the apparent involvement of a complex containing two Na ions and one glycine in the glycine-entry process (Vidaver, 1964) made this form of the hypothesis attractive.

No pumping can occur in the absence of an energy source. If a Na^+ gradient is the energy source for the glycine pump, in its absence glycine exit and entry rates from equal glycine concentrations must be equal, regardless of what the glycine concentrations are, and regardless of what the Na^+ concentrations are. This appears to be the case (Table IV).

Some other type of energy source, such as ATP, should be unequally distributed between the inside and outside of the cell. Also, a pump mechanism adapted to operate between the different phases, cell interior and plasma, might be expected to have a polarity. In either case unequal entry and exit rates might be expected under the conditions of the experiment shown in Table IV. However, it is possible to devise a pump model using, e.g., internal ATP as an energy source which would operate equally effectively in the two directions, thus these data do not prove the Na^+ operated pump hypothesis.[2] Since the relationships found between glycine pumping and a Na^+ gradient, and the occurrence of a complex containing both glycine and Na^+ are required by any pump model with a Na^+ gradient as energy source, but only correspond to a special case of the (e.g.) ATP-powered-pump hypothesis, they are taken to support the former.

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REFERENCES

Christensen, H. N., Riggs, T. R., Fischer, H., and Palatine, I. M. (1952b), *J. Biol. Chem. 198*, 1.
Christensen, H. N., Riggs, T. R., and Ray, N. E. (1952a), *J. Biol. Chem. 194*, 41.
Hempling, H. G., and Hare, D. (1961), *J. Biol. Chem. 236*, 2498.
Hoffman, J. F. (1958), *J. Gen. Physiol. 42*, 9.
Hoffman, J. F. (1962), *J. Gen. Physiol. 45*, 837.
Hoffman, J. F., Tosteson, D. C., and Whittam, R. (1960), *Nature 185*, 186.
Kromphardt, H., Grobeker, H., Ring, K., and Heinz, E. (1963), *Biochim. Biophys. Acta 74*, 549.
Riggs, T. R., Walker, L. M., and Christensen, H. N. (1958), *J. Biol. Chem. 233*, 1479.
Vidaver, G. A. (1964), *Biochemistry 3*, 662.
Whittam, R. (1962), *Biochem. J. 184*, 110.

[2] Such a model is represented by the sequence: $E_i + ATP \xrightarrow[\text{fast}]{} E_i^*$; $E_i^* \underset{\text{fast}}{\rightleftharpoons} E_o^*$; E_o^* (or E_i^*) + G_o (or G_i) + 2 Na_o^+ (or 2 Na_i^+) $\underset{\text{fast}}{\rightleftharpoons} E^*Na_2G_o$ (or $E^*Na_2G_i$); $E^*Na_2G_o$ (or $E^*Na_2G_i$) $\xrightarrow[\text{slow}]{} E^{**}G_o$ (or $E^{**}G_i$) + 2 Na_o^+ (or 2 Na_i^+); $E^{**}G_o$ (or $E^{**}G_i$) $\rightleftharpoons E^{**}G_i$ (or $E^{**}G_o$) (this is the translocation step); $E^{**}G_i$ (or $E^{**}G_o$) $\xrightarrow[\text{slow}]{} E_i$ (or E_o) + G_i (or G_o); $E_i \underset{\text{fast}}{\rightleftharpoons} E_o$. "E" is taken to be a mobile carrier in this model. The superscript asterisks represent "states" of E. "G" is glycine. All reactions except E_i + ATP → E_i^* might be catalyzed by the carrier ("E") itself and so be independent of location.

Mucate Inhibition of Glycine Entry into Pigeon Red Cells*

GEORGE A. VIDAVER†

From the Department of Chemistry, Indiana University, Bloomington

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A Donnan effect was produced in the pigeon-erythrocyte system when Cl^- was replaced by mucate ($COO^-(CHOH)_4COO^-$) in the incubation medium. The replacement of Cl^- by mucate caused a nearly complete inhibition of the Na^+-dependent component of glycine entry. The effect does not seem to be due to a specific "poisoning" by the mucate anion, but rather to the lack of external Cl^- and possibly also to some other concomitant (e.g., the electrical potential) of the Donnan effect. The inhibition is chiefly due to an increase in the glycine concentration giving half-maximal entry rate (K_m) of the entry process, although a moderate decrease in the maximum entry rate was also found. This effect of mucate is discussed in relation to Christensen's hypothesis that the Na^+ gradient may furnish the energy for amino acid–active transport.

Total glycine entry into pigeon red cells can be considered as consisting of two components, entry by a sodium-dependent route which obeys Michaelis-Menten kinetics with respect to both glycine and $(Na^+)^2$, and a small diffusionlike route. The Na^+ dependence implies the existence of a complex containing both Na^+ and glycine at some stage in the glycine-entry process (Vidaver, 1964a).

Experiments with hemolyzed and restored cells (Vidaver, 1964b) had supported Christensen's hypothesis that the difference in Na^+ concentration between the cell interior and the medium furnishes the energy for amino acid–active transport (Christensen *et al.*, 1952; Riggs *et al.*, 1958). Further tests, however, were necessary.

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† Part of this work was done during tenure of a U. S. Public Health Service postdoctoral fellowship.

TABLE I

EQUIVALENCE OF MUCATE AND TOLUENE-2,4-DISULFONATE EFFECTS AND THE RELIEF OF THEIR EFFECTS BY Cl^-, NO_3^-, AND ACETATE[a]

Monovalent Anion Added	Monovalent Anion Concentration (mM)	Mucate (mM)	Toluenedisulfonate (mM)	K^+ (mM)	Sucrose (mM)	Glycine Entry (μmoles/ml pellet H_2O in 15 min at 39°)
Cl^-	139	0	0	25	0	0.81
	0	57	0	0	95	0.17
Cl^-	30	57	0	30	45	0.45
Acetate$^-$	30	57	0	30	45	0.33
NO_3^-	30	57	0	30	45	0.34
	0	0	57	0	95	0.16
Cl^-	30	0	57	30	45	0.41
Acetate$^-$	30	0	57	30	45	0.28

[a] Cells were prepared, incubated, and processed as indicated under Methods. In all media, Na^+ was 129 mM and glycine was 0.5$_3$ mM. Monovalent anions were added to mucate and toluenedisulfonate media as K^+ salts, replacing part of the sucrose. The sum of meq phosphate plus Cl^-, mucate, and toluenedisulfonate, where present, equals the sum of Na^+ plus K^+ where present. The K^+ and sucrose concentrations are listed in the table but they are present only to preserve osmotic balance (see Discussion). The glycine-entry figures were not corrected for Na^+-independent glycine entry. (In this experiment, it was 0.05 μmole.)

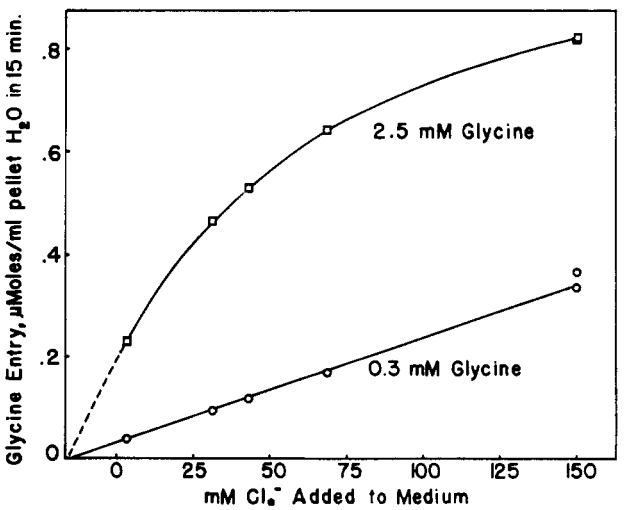

FIG. 1.—Glycine entry plotted against Cl^- added to the medium. Cells were prepared, incubated, and processed as indicated under Methods. In all media, Na^+ was 60 mM. Glycine concentrations are given on the face of the graph. The points at 150 mM Cl_o^- show entry from phosphate-buffered saline (60 mM Na^+, 105 mM K^+, 3 mM $H_2PO_4^-$, 6 mM HPO_4^{2-}, 150 mM Cl^-). The remaining points show entry from mucate media containing Na_2M, 28.5 mM; K_2M, 23.5 mM; KH_2PO_4, 3 mM; K_2HPO_4, 6 mM; sucrose, (121 − 1.83 × KCl) mM; KCl, (Cl^- indicated on graph − 3) mM; NaCl, 3 mM. The calculated value of the saline K_m is 0.4$_2$ mM.

This hypothesis requires that (some) Na^+ entry be coupled to glycine entry, i.e., that the energy in the Na^+ gradient be expended by the pumping of glycine. If the movement of this Na^+ were restricted or augmented, that of glycine should decrease or increase correspondingly.

The major anion in the usual media is Cl^-, to which the cell membrane is freely permeable. It is nearly impermeable to K^+ and Na^+. Therefore, if the Cl^- of the medium were replaced by a poorly penetrating anion, a Donnan effect should arise, and with it an electrical potential acting to oppose the entry and augment the exit of cations. Placing an electrical potential across the membrane must influence the free energy of the Na^+ gradient which, according to the hypothesis, powers the glycine pump. Since the Na^+ might cross the membrane as part of a complex containing more than just Na^+ and glycine (e.g., a "carrier," Cl^-, etc.), and the net charge of the complex might be positive, negative, or zero, glycine entry might be decreased, glycine exit increased, or both. Which of these changes would occur could not be predicted, but if the hypothesis is correct one or more of such changes must occur.

These considerations led to the testing of the effects of mucate ($COO^-(CHOH)_4COO^-$) on glycine entry.

It was found that substituting mucate for Cl^- strongly inhibited glycine entry. This effect was relieved by Cl^- in the presence of an unchanged concentration of mucate. It was not a direct "poisoning" by mucate. The "inhibition" resulted from effects on both the K_m and the V_{max} of the equation describing glycine entry, with K_m being more strongly affected.[1] Much of the effect is probably due to a Cl^- requirement for glycine entry since replacing both cell and medium Cl^- by methanesulfonate also strongly inhibits glycine entry. This inhibition was relieved by Cl^-.

MATERIALS AND METHODS

Pigeon red cells were prepared and incubated in centrifuge tubes (15 minutes, 39°), and [^{14}C]glycine entry was determined as previously described (Vidaver, 1964a). Radioactivity determinations were made on thin-sample plates.

Incubation media were phosphate buffered solutions (3 mM $H_2PO_4^-$, 6 mM HPO_4^{2-}) of alkali-metal mucates, sucrose, and alkali-metal chlorides. All media containing mucate also contained sucrose. All media contained 1.5 mg/ml glucose. Mucate-containing media were, formally, slightly hypotonic, but actually were effectively slightly hypertonic due to the Donnan effect. The compositions of the media varied with

[1] Abbreviations used in this work: K_m and V_{max} are, respectively, the glycine concentration giving half-maximal entry rate and the maximum entry rate, both as obtained from a Lineweaver-Burk plot. Although the terms are those of enzyme kinetics, their use is meant to imply only an analogy in kinetic behavior, not necessarily a detailed analogy of mechanism. The "saline K_m" is the K_m that would be found in a saline medium with the same Na^+ concentration as the "inhibited" medium being considered. Saline K_m values were calculated from a plot of K_m vs. $10^4/(Na^+)^2$ (Fig. 3, Vidaver, 1964a). K_2M and Na_2M are the dipotassium and disodium salts of mucic acid. The subscript "o" and "i" after a symbol for or name of a substance means the substance represented is present in the medium or the cell, respectively.

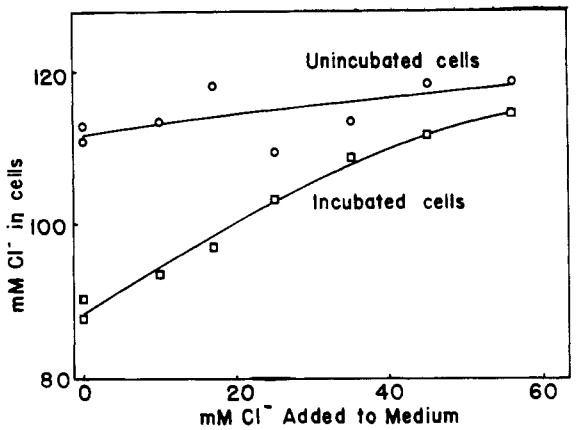

FIG. 2.—The chloride content of the cells, in μmoles/ml cell H_2O, plotted against Cl^- added to the medium. Values for cells suspended in media but not incubated are indicated by ⊙; for cells after 15-minute incubation at 39°, ⊡. Cells were prepared, incubated, and processed as indicated under Methods. All media contained 52 mM mucate, phosphates as for Figure 1, (105 − 1.86 × KCl) mM sucrose, and KCl equal to Cl^- indicated on the graph.

the experiment and are given in the tables and legends of the figures.

Stock solutions of Na_2M and K_2M (0.075 M or less) were prepared the day before use by neutralizing hot aqueous suspensions of mucic acid (Fisher, purified; recrystallized from water before use) with standardized KOH or NaOH. They were stored at room temperature.

The mucates of Ca^{2+}, Mg^{2+}, and K^+ range from nearly insoluble to sparingly soluble in that order. Therefore Ca^{2+} and Mg^{2+} were omitted from the media and K^+ was restricted. The precipitation of K_2M is slow. To avoid its precipitation from high-K^+ high-mucate media in the cold, the K_2M solutions at room temperature were mixed with the other components of the media and the mixture was chilled within one-half hour of use.

Chloride was determined in picric acid extracts by Hg^{2+} titration (Hawk *et al.*, 1954). Methanesulfonate solutions were prepared by neutralizing methanesulfonic acid (Eastman) with KOH or NaOH.

The dipotassium salt of toluene-2,4-disulfonic acid was prepared and recrystallized from aqueous ethanol (Blomstrand, 1872; Senhofer, 1872) and converted to the sodium salt by dismutation with $NaClO_4$. The Na^+ salt was precipitated with ethanol from the concentrated supernatant. The K^+ and Na^+ salts were standardized by flame photometry.

RESULTS

As shown in Table I, glycine entry was reduced when mucate replaced Cl^- in the medium. This reduction was relieved by adding back KCl at the expense of sucrose (i.e., with mucate held constant). The inhibition was also relieved by addition of KNO_3 or $KC_2H_3O_2$. Chloride was the most effective of these anions, with NO_3^- and acetate being less so and about equal to each other. The inhibition was not specific for mucate. Toluenedisulfonate had the same action and was similarly relieved by Cl^- and acetate.

The lesser effectiveness of NO_3^- compared with Cl^- is not owing to an inhibiting effect of NO_3^- superimposed on a relieving effect. Substitution of 50 out of 150 mM Cl^- by NO_3^- in a saline (i.e. mucate-free) medium had no effect on glycine entry.

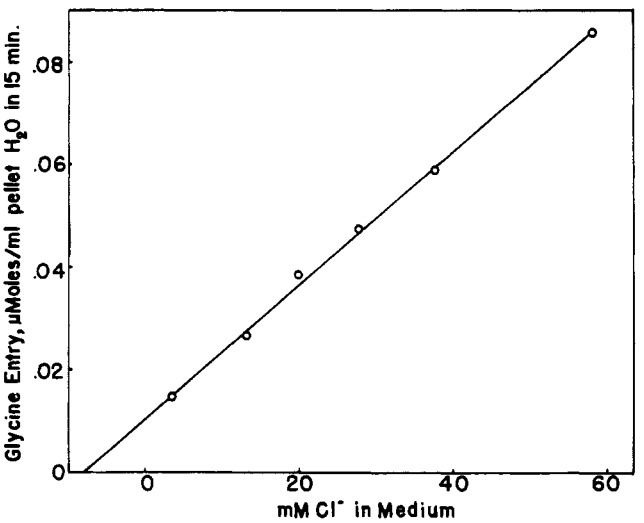

FIG. 3.—Glycine entry, with the Na^+-independent component subtracted, plotted against Cl^- in the medium. Values for Cl_o^- were obtained by adding together 50% of the amount of Cl^- lost from the cells to the medium during incubation (i.e., the time-averaged Cl^- contribution from cells to medium during incubation), all Cl^- contributed by the pellet to the medium before incubation, and the amount of Cl^- added to the medium as KCl. The greatest total contribution of Cl^- from cell pellets to media was 3.5 mM (lowest Cl^- point). For all samples, Na^+ was 44 mM, mucate 52 mM, glycine 0.4_2 mM. Sucrose was 105 − 1.86 × KCl added (in mM), and phosphates were as for Figure 1. The calculated saline K_m is 0.6_1 mM.

Figure 1 shows the relief of mucate inhibition by added Cl_o^-. In one curve the glycine concentration is somewhat lower than the (calculated) saline K_m value of 0.4 mM. In the other curve it is considerably higher. All points except those at the highest Cl_o^- are from media with the same mucate concentration. The highest Cl_o^- points show glycine entry from mucate-free (saline) medium. It can be seen that the saline points and the mucate points fall on the same curves. This indicates, as did the equivalence of mucate and toluenedisulfonate (Table I), that mucate inhibition is not merely a poisoning of the entry mechanism by mucate. The difference between the zero Cl^- values in Figure 1, lower curve, and in Table I may be ascribed to the $Na^+{}_o$ difference.

Figure 2 shows the variation of cell Cl^- with Cl_o^- in the mucate medium. The mucate concentration was held constant, with KCl added at the expense of the sucrose. The Donnan effect can be seen from the excess of Cl_i^- over Cl_o^-. The loss of Cl_i^- during incubation was presumably a consequence of withdrawal of cations from the cell by the Donnan-induced electrical potential. The plot of glycine entry vs. Cl_o^- in this experiment is shown in Figure 3. This differs from the low-glycine plot in Figure 1 in that the Cl_o^- contribution from the cells was taken into account, and also, in that the Na^+-dependent component (total entry minus entry from Na^+-free medium, Vidaver, 1964a) of glycine entry, rather than the total entry, was plotted. There appeared to be a small residue of Na^+-dependent glycine entry which was Cl_o^- independent.

Both the K_m and V_{max} terms of the equation describing Na^+-dependent entry (Vidaver, 1964a) are affected by mucate inhibition. As Figure 4 shows, K_m is greatly increased and V_{max} is moderately decreased. Both changes act to reduce glycine entry. This is presumably the reason for the difference between the high and low glycine curves in Figure 1.

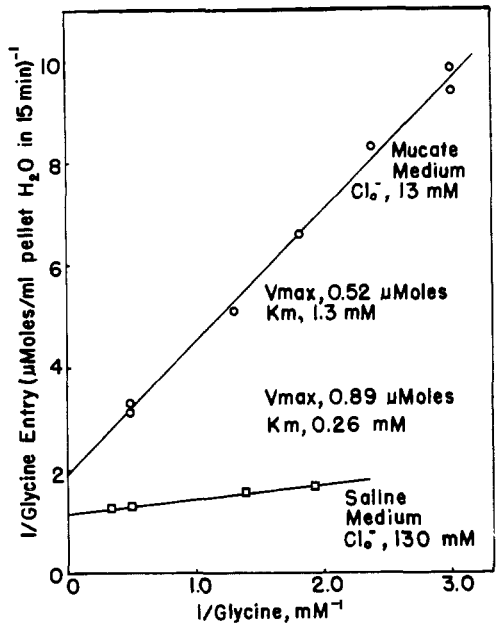

FIG. 4.—The Na^+-dependent components of glycine entry plotted in Lineweaver-Burk plots. ⊡, entry from saline medium; ⊙, entry from mucate medium with mucate inhibition partially relieved by 13.4 mM Cl_o^-. (Chloride, determined in the medium from unincubated cells, was 12.4 mM. The value of 13.4 was obtained by adding one-half the amount of Cl^- leaving the cells during incubation. This was taken to be 1 mM on the basis of earlier measurements of Cl^- loss [e.g., Fig. 2].) In the mucate medium, Na_2M was 60 mM; NaCl, 10 mM; Na_2HPO_4, 6 mM (total Na^+, 142 mM); KH_2PO_4, 3 mM; sucrose 80 mM. In the saline medium, NaCl was 130 mM; Na_2HPO_4, 6 mM (total Na^+, 142 mM); KH_2PO_4, 3 mM; sucrose, 24 mM. The values for Na^+-independent glycine entry from mucate and saline media were obtained from mucate and saline media, respectively, in which all Na^+ had been replaced by K^+. Glycine concentrations of 0.3 and 2 mM were used for the mucate values (the entry coefficients were the same) and 0.3 mM was used for the saline value. The Na^+-independent entry from mucate was somewhat less than from saline. Cells were prepared, incubated, and processed as indicated under Methods.

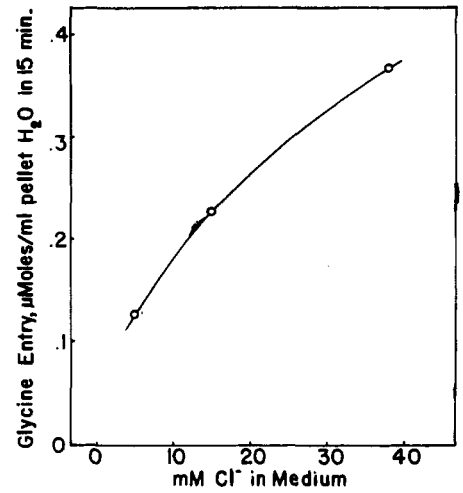

FIG. 5.—The Na^+-dependent component of glycine entry plotted against Cl_o^- in media in which the major anion was methanesulfonate. The cells were prepared as indicated under Methods and were then washed once with cold isotonic phosphate-buffered K^+-methanesulfonate, incubated 5 minutes at 39° with 1 volume 154 mM K^+-methanesulfonate, centrifuged, and then incubated for 10 minutes at 39° with 5.2 volumes of K^+-methanesulfonate. Chloride analysis showed these cells to have 4 μmoles Cl^-/ml cell H_2O. Aliquots of the cells were suspended in mixtures of methanesulfonate and Cl^- (sum: 142 mM) containing 3 mM $H_2PO_4^-$, 6 mM HPO_4^{2-}, 0.3_1 mM glycine, 104 (or zero) mM Na^+, and 53 (or 157) mM K^+. Incubation was for 20 minutes at 39°. The values plotted are 75% of the values obtained (to convert from μmoles/20 minutes to μmoles/15 minutes). Incubation procedure and further processing was as indicated under Methods. The calculated saline K_m value was 0.2_7 mM.

At a glycine concentration high relative to K_m the effect due to the K_m factor is masked.

As the data of Figure 5 illustrate, a considerable part of the mucate effect is merely a Cl^- requirement and is independent of a Donnan effect. When methanesulfonate replaced Cl_o^-, glycine entry was greatly inhibited. Adding back Cl_o^- at the expense of a small part of the methanesulfonate relieved this inhibition. In the experiment shown in Figure 5, the cell Cl^- had been exchanged for methanesulfonate before the experimental incubation, so the Donnan effect was absent during glycine entry.

DISCUSSION

Earlier work (Vidaver, 1964a) had indicated that variations of sucrose and K^+ concentrations had no effect on glycine entry. The effects on glycine entry produced by (isoosmotic) substitution of K^+ salts for sucrose in mucate media are therefore presumed due only to the effects of the added anions.

The mucate inhibition does not seem to be caused by combination of mucate with any component of the cell membrane or glycine "pump." If it were, the chemically quite different toluenedisulfonate should have at least quantitatively different effects than mucate. In addition, such an action should result in a difference between a glycine entry vs. Cl_o^- curve with mucate present, and such a curve with mucate absent. This is contrary to the observations in Figure 1, where the saline points and the mucate points fall on the same curves.

The near constancy of cell Cl^- over a range of Cl_o^- values (Figure 2) shows the impermeability of the cells to mucate and the existence of the Donnan effect. A Donnan effect might affect glycine entry in at least four ways. First, if glycine crosses the membrane in company with two Na ions, the complex might have a net positive charge and therefore be restrained by the electrical potential accompanying the Donnan effect. Such an action would decrease V_{max}. However this need not happen since the counter ions, loosely associated with the complex in an aqueous phase, might become more closely associated with it if the complex entered a phase with a low dielectric constant. In that case, the net charge of the complex within the membrane could be effectively zero.

Second, the K_m might be affected. If a mobile carrier is assumed, it must return to the outside of the membrane, free of glycine and possibly of Na^+. If such an "empty" carrier had a net negative charge, its return would be impeded by the electrical potential. If the transit rate of the empty carrier were rapid compared to the "loaded" one, such an effect would appear as an increase in K_m. This is because, under the foregoing assumptions, the term "E" in equation (1) of Vidaver (1964a) would be replaced by $(1 + K_\alpha)\ E_o$, where $K_\alpha = E_i/E_o$; K_α would increase with the Donnan potential; and K_α would appear in the final equation in the group of terms representing K_m for glycine.

Third, an effect on V_{max} might also arise from changes

in the bulk properties of the membrane (e.g., resistance to motion of molecules in it) due to polarization of dipolar molecules in it.

Fourth, replacement of Cl_o^- by mucate could also reduce glycine entry if there were a specific Cl^- requirement for formation of the glycine-containing complex (a K_m effect) or for its passage across the membrane (a V_{max} effect). Of the various possible mechanisms, the present work provides direct evidence only for the last.

Low concentrations of Cl_o^- seem more effective in relieving methanesulfonate inhibition than in relieving mucate inhibition (e.g., compare glycine entry at 10 mM Cl_o^- in Fig. 5 with that in Fig. 1 or 3). Although the point would have to be established by direct comparison at identical Na_o^+ and $glycine_o$ values, the difference is probably too great to be due solely to the differences in Na_o^+ and in the $glycine_o$/saline K_m ratios; therefore mucate inhibition includes, but is probably more than, a specific requirement for Cl_o^-.

The prediction from the hypothesis (see introductory paragraphs) was that the Donnan effect should reduce glycine entry relative to exit. This prediction applies to the special case (not attainable with intact cells) where Na_i^+ equals Na_o^+ and $glycine_i$ equals $glycine_o$. With the present experiments, only the effect on entry was measured. While the observed inhibition of entry is consistent with the hypothesis that the Na^+ gradient is the energy source for the glycine pump, it does not prove it.

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REFERENCES

Blomstrand, C. W. (1872), *Chem. Ber. 5*, 1084.
Christensen, H. N., Riggs, T. R., Fischer, H., and Palatine, I. M. (1952), *J. Biol. Chem. 198*, 1.
Hawk, P. B., Oser, B. L., and Summerson, W. H. (1954), Practical Physiological Chemistry, 13th ed., Blakiston, New York, p. 626.
Riggs, T. R., Walker, L. M., and Christensen, H. N. (1958), *J. Biol. Chem. 233*, 1479.
Senhofer, C. (1872), *Ann. 164*, 126.
Vidaver, G. A. (1964a), *Biochemistry 3*, 662.
Vidaver, G. A. (1964b), *Biochemistry 3*, 795 (preceding paper, this issue).

Some Tests of the Hypothesis that the Sodium-Ion Gradient Furnishes the Energy for Glycine-active Transport by Pigeon Red Cells*

GEORGE A. VIDAVER

From the Department of Chemistry, Indiana University, Bloomington

Received January 9, 1964

Three further tests of the Na^+-gradient hypothesis are applied. These, like one used earlier, support the hypothesis, which is therefore considered to be established. The findings with the three new tests are as follows. (1) Two Na ions enter the cells in concert with one glycine, as expected from the previously reported kinetic dependence of glycine entry on $(Na^+)^2$. (2) A system with high but equal concentrations of Na^+ inside and out, which does not pump glycine due to the absence of a Na^+ gradient, can be caused to pump glycine (out) by a Donnan effect. In the presence of the Donnan electrical potential there is a Na^+-electrochemical gradient even though there is no Na^+-concentration gradient. (3) No correlation is found between the concentration of cell nucleotide polyphosphate(s) (ATP) and glycine-pump activity.

Total glycine entry into pigeon red cells can be considered to consist of two components: entry by a sodium-dependent route which obeys Michaelis-Menten kinetics with respect to both glycine and $(Na^+)^2$, and a small diffusionlike route. The Na^+ dependence implies the existence of a complex containing both Na^+ and glycine at some stage in the entry process. Part of the glycine exit from Na^+-enriched cells is also Na^+ dependent (Vidaver, 1964a).

Pigeon red cells, like mammalian red cells, can be hemolyzed and restored (made again selectively permeable). The cation and glycine concentrations in the restored cells are largely determined by the cation and glycine concentrations in the lysing and restoring solutions. Such preparations can pump glycine, but only if a sodium gradient exists (Vidaver, 1964b) These experiments with lysed and restored cells supported Christensen's hypothesis that the difference in Na^+ concentration between the cell interior and medium furnishes the energy for amino acid–active transport (Christensen *et al.*, 1952; Riggs *et al.*, 1958).

Several predictions made from the hypothesis could be used to test it. If energy comes from the Na^+ gradient, the energy in it must be expended; that is, Na^+ must move down its gradient, and some of this movement must be coupled to glycine movement against the glycine gradient. From the kinetic dependence of glycine entry on $(Na^+)^2$, two Na ions would be expected to enter the cell for every glycine entering by the Na^+ dependent route. The test of this prediction will be referred to as the "stochiometry test."

It had been found that replacement of Cl^- of the medium by mucate produced a Donnan effect with its accompanying electrical potential (Vidaver, 1964c). Hemolyzed and restored cells with equal internal and external concentrations of both Na^+ and glycine

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